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Use of Bacteriophage T7 RNA Polymerase to Direct Selective High-level Expression of Cloned Genes

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A gene expression system based on bacteriophage T7 RNA polymerase has been developed. T7 RNA polymerase is highly selective for its own promoters, which do not occur naturally in *Escherichia coli*. A relatively small amount of T7 RNA polymerase provided from a cloned copy of T7 gene 1 is sufficient to direct high-level transcription from a T7 promoter in a multicopy plasmid. Such transcription can proceed several times around the plasmid without terminating, and can be so active that transcription by *E. coli* RNA polymerase is greatly decreased. When a cleavage site for RNase III is introduced, discrete RNAs of plasmid length can accumulate. The natural transcription terminator from T7 DNA also works effectively in the plasmid. Both the rate of synthesis and the accumulation of RNA directed by T7 RNA polymerase can reach levels comparable with those for ribosomal RNAs in a normal cell. These high levels of accumulation suggest that the RNAs are relatively stable, perhaps in part because their great length and/or stem-and-loop structures at their 3' ends help to protect them against exonucleolytic degradation. It seems likely that a specific mRNA produced by T7 RNA polymerase can rapidly saturate the translational machinery of *E. coli*, so that the rate of protein synthesis from such an mRNA will depend primarily on the efficiency of its translation. When the mRNA is efficiently translated, a target protein can accumulate to greater than 50% of the total cell protein in three hours or less. We have used two ways to deliver active T7 RNA polymerase to the cell; infection by a lambda derivative that carries gene 1, or induction of a chromosomal copy of gene 1 under control of the *lacUV5* promoter. When gene 1 is delivered by infection, very toxic target genes can be maintained silent in the cell until T7 RNA polymerase is introduced, when they rapidly become expressed at high levels. When gene 1 is resident in the chromosome, even the very low basal levels of T7 RNA polymerase present in the uninduced cell can prevent the establishment of plasmids carrying toxic target genes, or make the plasmid unstable. But if the target plasmid can be maintained, induction of chromosomal gene 1 can be a convenient way to produce large amounts of target RNA and/or protein. T7 RNA polymerase seems to be capable of transcribing almost any DNA linked to a T7 promoter, so the T7 expression system should be capable of transcribing almost any gene or its complement in *E. coli*. We expect that comparable T7 expression systems can be developed in other types of cell.

1. Introduction

The RNA polymerase of bacteriophage T7 has a stringent specificity for its own promoters (Chamberlin *et al.*, 1970). Seventeen such promoters are found in T7 DNA, and they contain a highly conserved nucleotide sequence extending from -17 to +6 relative to the start of the RNA chain (Oakley & Coleman, 1977; Rosa, 1979; Panayotatos

& Wells, 1979; Dunn & Studier, 1983). The conserved sequence required to make an active promoter for T7 RNA polymerase is long enough that it is unlikely to occur by chance in any DNA unrelated to T7 DNA, and no T7 promoters are known to be present in the DNA of *Escherichia coli*, a natural host. T7 uses its highly selective polymerase to direct transcription to its own DNA rather than to host DNA during infection.

Transcription by T7 RNA polymerase is very active as well as very selective. A single-chain enzyme with a molecular weight close to 100,000,

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T7 RNA polymerase initiates RNA chains very efficiently, and elongates them about five times faster than does *E. coli* RNA polymerase (Chamberlin *et al.*, 1970; Chamberlin & Ring, 1973; Golomb & Chamberlin, 1974). Amber mutations do not seem to have polar effects on transcription by T7 RNA polymerase during T7 infection (Studier, 1972), suggesting that the enzyme may not be subject to the factors that cause termination of transcription by *E. coli* RNA polymerase (reviewed by Holmes *et al.*, 1983). Furthermore, the one terminator for T7 RNA polymerase in T7 DNA is not completely efficient (Carter *et al.*, 1981), and terminators that have been observed in unrelated DNAs are much less efficient (McAllister *et al.*, 1981; J. J. Dunn, personal communication). Therefore, T7 RNA polymerase should be capable of efficiently producing complete transcripts from almost any DNA that is linked to a T7 promoter.

Its selectivity, activity and ability to produce complete transcripts make T7 RNA polymerase attractive to use for directing high-level expression of selected genes in *E. coli*, and perhaps in other types of cell as well. Since T7 promoters are not utilized by *E. coli* RNA polymerase (nor presumably by the RNA polymerases of any other cell), the presence of a T7 promoter in the cell should by itself have little effect on gene expression. However, introduction of active T7 RNA polymerase into such a cell should induce active and selective transcription from the T7 promoter. The problem in designing a high-level expression system then becomes how to deliver active T7 RNA polymerase to a cell that contains a T7 promoter.

Infection by T7 itself has been the only way to deliver T7 RNA polymerase to *E. coli* cells. Indeed, the T7 RNA polymerase made during T7 infection directs the expression of genes under the control of T7 promoters in plasmids (Campbell *et al.*, 1978; Studier & Rosenberg, 1981; McAllister *et al.*, 1981). However, these gene products do not accumulate to high levels, because there is competition from promoters in T7 DNA and the T7 infection quickly kills the cell. With the cloning of T7 gene 1, the gene for T7 RNA polymerase (Davanloo *et al.*, 1984), a source of active enzyme independent of T7 infection and free of competing T7 promoters became available. In this paper, we describe the use of T7 RNA polymerase from the cloned gene to direct sustained, high-level expression of selected genes in *E. coli*. Tabor & Richardson (1985) have also cloned gene 1 and developed a high-level expression system based on T7 RNA polymerase.

2. Materials and Methods

(a) Bacteria, plasmids and phage strains

The host for growing lambda derivatives was ED8739 (F^- *metB* *hsdS* *supE* *supF*; Borek *et al.*, 1976), which is a *supF* derivative of 803 (Wood, 1966) and was obtained from N. Murray by way of J. Sambrook and B. Burr. HMS174 (F^- *hsdR* *recA* *Rif^R*; Campbell *et al.*, 1978) and BL21 (F^- *hsdS* *gal*), derived from B834 (Wood, 1966) by

Table 1
Plasmids used in this work

Plasmids	Genetic elements inserted	Ref.
pBR322		1
pAR1151	1	2
pAR1219	<i>lacI-lacUV5-1</i>	2
pAR951	$\phi 10$	3
pAR1494	$\phi 10$ (clockwise)	3
pAR946	$\phi 10$ -R0-3-0-3	3
pAR219	2- $\phi 2$ -5	4
pAR511	$\phi 2$ -5-2-5	3
pAR1012	R4.7- $\phi 4$ -7-4-7-5	3
pAR525	6-6-3- $\phi 6$ -5-R6-5-6-5	3
pAR213	8- $\phi 9$ -(9) (243 aa)	4
pAR441	$\phi 9$ -9- $\phi 10$	3
pAR436	$\phi 10$ -10-T ϕ	4

All inserts are in the *Bam*HI site of pBR322 and all, except in pAR1494, are oriented so that transcription from T7 promoters, or transcription that produces T7 mRNAs, proceed counter-clockwise. The order of T7 promoters, intact T7 genes, any RNase-III cleavage sites (R0-3 and R6-5), and the T ϕ terminator in the inserted fragments is given (for more details about these genes and genetic signals, see Dunn & Studier, 1983). Representations of pAR951, pAR1494 and pAR441 are given in Fig. 2, and the sizes and functions of proteins specified by the plasmids are given in Table 2. Plasmid pAR213 carries all of gene 8 and a large fragment of gene 9, predicted to direct a T7-pBR322 fusion protein that is 243 amino acid residues (aa) long. References are: 1, Bolivar *et al.* (1977); 2, Davanloo *et al.* (1984); 3, constructed in this laboratory and to be described in detail elsewhere; 4, Studier & Rosenberg (1981).

transduction to Met⁺, are non-suppressing hosts. ED8739 is $r_K^- m_K^-$; HMS174 is $r_K^- m_K^-$; and BL21 is $r_B^- m_B^-$. Bacterial strains, with or without plasmids, are stored at -80°C after mixing a sample of growing or saturated culture with 0.1 vol. 80% (v/v) glycerol.

Plasmids are listed in Table 1 and were all derived from pBR322 (Bolivar *et al.*, 1977) by inserting fragments of T7 DNA, or of T7 DNA plus *lac* DNA, into the *Bam*HI site. Sizes and functions of proteins that can be expressed from these plasmids under control of a T7 promoter are given in Table 2.

The lambda cloning vector D69 (Mizusawa & Ward, 1982) was obtained from D. Court, and lambda *cI857ind1Sam7* was obtained from R. Davis. Deletion 4107 is a T7 deletion mutant isolated in this laboratory. It arose by a crossover between homologies in the RNase-III cleavage sites ahead of genes 0-5 and 1-1 of T7, which removed all of genes 0-5, 0-6, 0-7 and 1, about 11.1% of the DNA.

(b) Growth media

Complex growth media include ZB medium (10 g N-Z-amine A/l and 5 g NaCl/l), ZY medium (10 g N-Z-amine A/l, 5 g Bacto yeast extract/l and 5 g NaCl/l) or M9ZB medium, which contains the components of both M9 and ZB media. N-Z-amine A was from Sheffield Products (P.O. Box 398, Memphis, TN 38101) and yeast extract was from Difco. Media in which N-Z-amine A is replaced by Bacto Tryptone (Difco) have given essentially equivalent results. Defined growth media included M9 medium (1 g NH₄Cl/l, 3 g KH₂PO₄/l, 6 g Na₂HPO₄/l, 4 g glucose/l and 1 ml 1 M-MgSO₄/l) and B2 medium, which is essentially M9 medium in which all but 0.16 mM of the phosphate is replaced by salts and bis-Tris buffer (Studier, 1975). M9 maltose or B2 maltose are the

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Table 2
Sizes and functions of proteins specified by plasmids

Gene	No. of amino acids	M_r	Function	Ref.
A. For pBR322 proteins				
<i>bla</i> precursor	285	31,393		1
<i>bla</i> processed	263	28,899	β -Lactamase	1
<i>rop</i>	63	7226	Control of replication	2
B. For T7 proteins				
0-3	116	13,678	Anti-restriction	3
2	63	7043	Anti- <i>E. coli</i> RNA polymerase	3
2-5	231	25,562	DNA-binding	3
4-7	135	15,208	Unknown	3
5	704	79,692	DNA polymerase	3
6	347	39,995	Exonuclease	3
6-3	37	4088	Unknown	3
6-5	84	9474	Unknown	3
8	535	58,989	Head-tail junction	3
9	306	33,766	Head assembly	3
10A	344	36,414	Major head protein	3
10B	397	41,800	Minor head protein	3

It is assumed that the *bla* precursor does not retain the initial methionine residue but that the *rop* protein does. The gene *10B* protein of T7 is produced by frameshifting during translation of the *10A* mRNA (Dunn & Studier, 1983). The relative mobilities of T7 proteins in gel electrophoresis in the presence of sodium dodecyl sulfate do not always correspond to their relative molecular weights; see Fig. 3 of Dunn & Studier (1983) for an electrophoretic pattern in which the relative positions of many T7 proteins are identified. References are: 1, Sutcliffe (1979); 2, Cesareni *et al.* (1982) and Som & Tomizawa (1983); 3, Dunn & Studier, 1983.

equivalent media, in which glucose is replaced by maltose. When growing plasmid-containing cells, ampicillin was added to the medium, usually at a concentration of 20 μ g/ml, but as high as 200 μ g/ml. Dilutions of bacteria or phage for titering were made in ZB medium, and samples were plated by mixing with 2.5 ml of melted top agar (0.7% (w/v) agar in ZB medium), and spreading on plates containing 20 ml of hardened bottom agar (1% agar in ZB medium).

(c) Growth and manipulation of lambda derivatives

General procedures for working with lambda are described by Maniatis *et al.* (1982) and Hendrix *et al.* (1983); we used the following specific procedures. Phage stocks were grown in ZY medium. Stock lysates were grown by adding a single plaque and 50 μ l of a fresh overnight culture of ED8739 to 35 ml of growth medium in a 125-ml flask, and shaking at 37°C until lysis; larger volumes were grown by adding 10 μ l of lysate and 1 ml of cells to 500 ml of medium in a 1 liter flask. Lysates typically contained a few times 10^{10} infective phage particles/ml.

Phage were purified by precipitation with polyethylene glycol followed by rapid isopycnic banding in CsCl step gradients. All solutions used during purification, including CsCl solutions, contained 10 mM-Tris·HCl (pH 8.0), 10 mM-MgSO₄, 100 μ g gelatin/ml to keep the phage intact. The purified phage were stored in the CsCl solution, and dilutions were made in 0.1 M-NaCl, 50 mM-Tris·HCl (pH 8.0), 10 mM-MgSO₄, 100 μ g gelatin/ml.

Plating bacteria for titrating lambda derivatives were grown overnight in ZB medium, collected by centrifuga-

tion, resuspended in the same volume of 10 mM-MgSO₄, and left at 4°C until use, usually within 2 days. An appropriate dilution of phage in ZB medium was mixed with 0.1 ml of plating bacteria and incubated for 10 min at 37°C to allow adsorption before plating.

Genetic crosses were made in ED8739 by adsorbing 5 infectious particles of each parental phage per cell for 10 min at 37°C in 10 mM-MgSO₄, diluting in 10 vol. ZY medium, and shaking at 37°C until lysis. Recombinants were screened for the presence of an expressible gene for T7 RNA polymerase by inability to grow on a lawn of ED8739/pAR511, which contains T7 gene 2.5 under control of a T7 promoter; transcription by T7 RNA polymerase produced during infection prevents lambda from forming a plaque.

(d) Cloning into D69

The cloning vector D69, a lambda derivative that has *imm*²¹ and a single *Bam*HI cloning site within the *int* gene, was used essentially as described (Mizusawa & Ward, 1982). Restriction endonucleases and enzymes used in cloning DNA were obtained from New England Biolabs. A mixture of 500 ng of a *Bam*HI digest of D69 DNA and a 5-fold molar excess of a *Bam*HI fragment that contained the gene for T7 RNA polymerase (purified from pAR1151 or pAR1219 (Davanloo *et al.*, 1984)) was ligated with phage T4 DNA ligase and packaged in a lambda packaging system kindly provided by B. Burr. About 90% of the resulting plaques had inserts, and both orientations were obtained. The genetic composition of D69 and the derivatives used in this work are given in Table 3 and Fig. 1.

(e) Lysogens of D69 derivatives

Cloning into the *Bam*HI site of D69 interrupts the *int* gene, which is needed for integration into the chromosome; so, in order to make lysogens of these phages, *int* function was provided from a lysogen of heterologous immunity. A drop of lysate was spotted onto a lawn of the helper lysogen, the center of the cleared spot was used to grow a culture, and individual colonies from the culture were tested for the appropriate immunity or for the presence of a functional cloned gene.

(f) Directed expression of target genes, where T7 RNA polymerase is provided by CE6 infection

Under appropriate conditions, each of the D69 derivatives listed in Table 3, except DE1, is capable of making enough T7 RNA polymerase during infection to generate high-level expression of target genes cloned in a plasmid under control of a T7 promoter. We have usually used CE6, and the following procedures have been successful in obtaining efficient infection and high-level expression.

Cultures are grown at 37°C, usually in a shaking incubator or waterbath, and plasmid-containing cells are grown in the presence of ampicillin, usually at 20 μ g/ml. In order to obtain efficient infection (as measured by loss of colony-forming units), cultures are grown in the presence of maltose (and no glucose) to induce the lambda receptor (Schwartz, 1967). When the multiplicity of infection is higher than about 20 infectious phage particles per cell, the overall rate of protein synthesis after infection is drastically reduced and little protein is produced from the target gene (see Results, section (b)(i), and Fig. 3). Therefore, we aim for multiplicities in the

range 5 to 10, where both infection and protein synthesis are efficient.

Most experiments have been done with cells grown in M9 maltose, where rates of protein synthesis can be measured by pulse-labeling with [35 S]methionine. When the absorbance at 600 nm (A_{600}) of an actively growing culture reaches 0.3, glucose is added to give a concentration of 4 mg/ml, and the culture is grown for an additional 1 to 2 h, during which the A_{600} reaches 0.6 to 1 and the cell concentration is typically 5×10^8 /ml. At this point, $MgSO_4$ is added to a final concentration of 10 mM, and purified CE6 phage is added to a final concentration of 2×10^9 to 4×10^9 /ml (0.001 volume of a stock having an A_{260} of 6). The multiplicity of infection is typically around 7, and the efficiency of infection is typically greater than 95%, as measured by loss of colony-forming ability. Addition of glucose and 10 mM- $MgSO_4$ to the medium is not necessary, but seems to give slightly better production of protein from the target genes. Cells are usually harvested 3 h after infection, enough time for substantial accumulation of target protein but not enough time for uninfected cells to overgrow the culture.

When RNA synthesis is to be analyzed by pulse-labeling with $^{32}PO_4$, the same protocol is followed, except that the cells are grown in B2 maltose. Efficient infection of cultures and successful expression of target genes has been obtained in ZY medium supplemented with 0.4% (w/v) maltose. This medium can be used where a rich medium is necessary or desirable. When using ZY medium, no glucose or $MgSO_4$ has been added.

(g) Directed expression of target genes, where T7 RNA polymerase is provided by induction of a chromosomal copy of the gene

The lysogen BL21(DE3) contains a single copy of the gene for T7 RNA polymerase in the chromosome under control of the inducible *lacUV5* promoter (Table 3, Fig. 1). As discussed in Results (section (c)(iii)), some T7 RNA polymerase is produced from the prophage even in the absence of added inducer, so plasmids containing target genes that are toxic can be difficult or impossible to maintain in BL21(DE3). However, when the plasmid can be maintained, addition of IPTG† induces the *lacUV5* promoter to produce T7 RNA polymerase, which in turn initiates high-level expression of the target gene in the plasmid. The level of expression is usually comparable with that found upon infection with CE6.

Because toxicity of the target gene can lead to loss of plasmid or accumulation of non-functional mutants (Results, section (c)(iii)), cultures are sampled just before induction and titered for viable cells on plates without additives or in the presence of 0.5 mg of ampicillin, 2.5 μ mol of IPTG, or both, added to the top agar. BL21(DE3) carrying a plasmid without a T7 promoter will form colonies on each of these plates, as will non-functional mutants that retain plasmid. In a typical culture useful for producing target proteins, almost all cells will form colonies on plates without additives or containing only ampicillin, less than 2% of the cells will form a colony on plates containing only IPTG, and less than 0.01% will form a colony on plates containing both ampicillin and IPTG.

BL21(DE3) has been used for most experiments where T7 RNA polymerase is supplied by induction, and

cultures have been grown in M9, M9ZB, ZY medium or ZY medium supplemented with 0.4% (w/v) glucose. Induction is with 0.4 mM-IPTG when the culture reaches an A_{600} of 1, corresponding to about 5×10^8 to 10^9 viable cells per ml. Cells are typically harvested 3 h after induction, after substantial accumulation of target protein is achieved but before the culture can be overgrown with cells that have lost plasmid or are otherwise unproductive.

3. Results

Cloned T7 gene 1 (Davanloo *et al.*, 1984) provides a source of T7 RNA polymerase for directing selective, high-level expression of target genes under control of a T7 promoter. In attempting to exploit the enzyme in this way, we first tried to put all of the elements needed for expression of the target gene in a plasmid: starting with plasmid pAR1219, which contains gene 1 under control of the inducible *lacUV5* promoter, we tried to add target genes under control of a T7 promoter. These efforts were unsuccessful, apparently because the levels of active T7 RNA polymerase in the uninduced state were such that plasmids could not be maintained. This was not too surprising, since uninduced pAR1219 produces enough active T7 RNA polymerase to complement T7 mutants defective in gene 1 (Davanloo *et al.*, 1984), which should be enough to generate substantial transcription from a T7 promoter in the plasmid.

T7 RNA polymerase is so active and selective that we considered it might be difficult to find conditions where cloned gene 1 would be shut off to such a degree that the cell could tolerate diverse target genes, many of which might be toxic. Therefore, to establish the potential of T7 RNA polymerase to direct expression, we decided to remove gene 1 from the cell entirely, and to introduce it only at the time we wished it to be active. Placing the cloned gene 1 into a phage lambda vector and introducing the gene by lambda infection seemed a convenient way to do this. Lambda itself carries no T7 promoters, and gene 1 could be transcribed by *E. coli* RNA polymerase from promoters normally in lambda DNA or from an *E. coli* promoter cloned with the gene. Furthermore, expression of the lambda genes can be prevented by infecting an immune host (although this proved to be unnecessary), and lambda can be used to insert a single copy of the cloned gene 1 into the chromosome simply by forming a lysogen.

In the complete absence of T7 RNA polymerase, even genes whose products are very toxic to the cell might be maintained stably behind a T7 promoter if cloned in a site where transcription by *E. coli* RNA polymerase is low enough. Almost all of the genes from T7 DNA, even those expected to be very toxic, have been cloned in the *Bam*HI site of the plasmid pBR322 in the orientation where transcription in the counterclockwise direction is required to produce mRNA, even though genes in this site are expressed to some extent (Studier & Rosenberg, 1981; Davanloo *et al.*, 1984; and unpublished

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CE2	T
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† Abbreviation used: IPTG, isopropyl- β -D-thiogalactopyranoside.

results). Some of these genes were cloned with a nearby T7 promoter in the same fragment of T7 DNA, and others have been cloned behind a T7 promoter from a different part of T7 DNA. We have used this collection of cloned genes and associated T7 promoters to test how effectively T7 RNA polymerase produced from the cloned gene can induce sustained, high-level expression of selected target genes.

(a) Construction of lambda derivatives that carry the T7 RNA polymerase gene

We placed the active gene for T7 RNA polymerase, alone or under control of the *lacUV5* promoter, in the *Bam*HI site of the lambda cloning vector D69 (Fig. 1 and Table 3). In one orientation, represented by DE1, no *E. coli* promoters that would direct transcription of gene 1 mRNA are known to be present in D69 DNA; DE3 contains gene 1 in the same orientation but under control of the *lacUV5* promoter. In the opposite orientation, represented by DE2, the p_L and p_i promoters of D69 can direct transcription of gene 1 mRNA; DE4 contains gene 1 in this orientation but is also under control of the *lacUV5* promoter. All four of these strains grow well and produce high-titer lysates, and all have been obtained as lysogens of HMS174 and BL21. Apparently, the presence of the T7 RNA polymerase gene, whether expressed or not, has little effect on lambda growth or lysogeny in the usual hosts.

Further derivatives carrying gene 1 were obtained by crossing DE2 (in which gene 1 is under

Table 3
Derivatives of the lambda cloning vector D69 that carry T7 gene 1

Phage	Fragment cloned into <i>int</i>	Orientation	Immunity	<i>nin5</i>	<i>Sam7</i>
D69	None		21	Δ	+
DE1	T7 gene 1	S	21	Δ	+
DE2	T7 gene 1	E	21	Δ	+
DE3	<i>lacUV5</i> -gene 1	S	21	Δ	+
DE4	<i>lacUV5</i> -gene 1	E	21	Δ	+
DE6	T7 gene 1	E	21	+	<i>am</i>
CE2	T7 gene 1	E	λ C1857	+	+
CE6	T7 gene 1	E	λ C1857	+	<i>am</i>

D69 is described by Mizusawa & Ward (1982). All phages in the Table contain both a mutation that eliminates the *Bam*HI site at nucleotide 5505 of lambda DNA and a deletion of the DNA between the *Eco*RI sites at nucleotides 21,226 and 26,104. (Locations in lambda DNA are taken from the compilation by Hendrix *et al.*, 1983.) The cloning site in D69 is the *Bam*HI site at nucleotide 27,972 in the *int* gene; the fragments inserted were from plasmid pAR1151 (gene 1 itself) or from pAR1219 (gene 1 under control of the *lacUV5* promoter; Davanloo *et al.*, 1984). The orientation in which mRNA for T7 RNA polymerase would be transcribed from the p_L and p_i promoters of the phage (see Fig. 1) is designated E (for expressed); the opposite orientation is designated S (for silent). The immunity region between nucleotides 34,379 and 38,617 either has been replaced by the immunity region of phage 21 or is the lambda immunity region having the C1857 and *ind1* mutations. The *nin5* deletion (Δ) removes nucleotides 40,502 to 43,307.

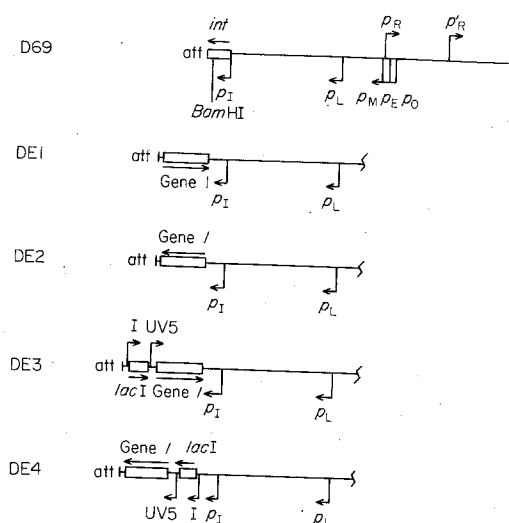


Figure 1. Locations and orientations of *E. coli* promoters and inserted genes in D69, DE1, DE2, DE3 and DE4. These phages are described in Table 3. The DNA between the attachment site (*att*) and the right end of the mature phage DNA is represented (for a map of the entire lambda DNA molecule see page 473 of Hendrix *et al.*, 1983). The locations of known *E. coli* promoters and the *Bam*HI cloning site within the *int* gene of D69, and the locations and orientations of the *lacI* promoter, the *lacI* gene, the *lacUV5* promoter, and T7 gene 1 in the different derivatives of D69 are represented to scale.

control of p_L and p_i) with lambda *cI857ind1Sam7*. The *cI857* mutation would make expression from the p_L promoter temperature-inducible, and the *Sam7* mutation would prevent lysis of the infected or induced cells. Three recombinants selected for further use all appear to have retained the left arm of DE2 and to have lost the *nin5* deletion: DE6 retains *imm*²¹ and has acquired the *Sam7* mutation; CE2 has acquired *imm* ^{λ} (*cI857ind1*); and CE6 has acquired both *imm* ^{λ} (*cI857ind1*) and *Sam7* (Table 3).

(b) Selective expression of target genes when T7 RNA polymerase is provided by lambda infection

(i) Optimization of expression

Conditions for optimal expression of target genes during infection by the lambda derivatives carrying gene 1 were worked out largely using plasmid pAR441 in HMS174. Plasmid pAR441 carries a fragment of T7 DNA containing the $\phi 9$ promoter for T7 RNA polymerase, gene 9, and the $\phi 10$ promoter, oriented so that the T7 promoters direct the synthesis of mRNA for the β -lactamase of pBR322 as well as for the gene 9 protein (Fig. 2). The gene 9 protein is made in fairly large amounts during T7 infection and is involved in assembly of the T7 capsid (Studier, 1972).

The first problem was to obtain reproducible, efficient infection by the phages. Synthesis of the lambda receptor is repressed when cells are grown

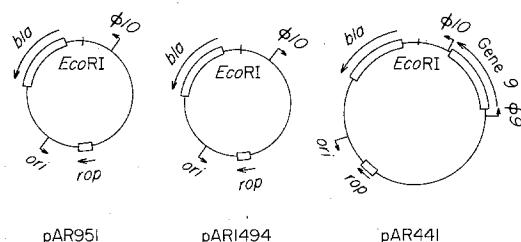


Figure 2. Locations and orientations of coding sequences and T7 promoters in pAR951, pAR1494 and pAR441.

in the presence of glucose, but is induced in the presence of maltose and the absence of glucose in both minimal and rich media (Schwartz, 1967). Good expression of target genes can be obtained even in cells that have grown in the presence of glucose, but the 20% to 30% of cells that typically escape infection give a rather high background in pulse-labeling experiments and will rapidly

overgrow the culture. Procedures optimized both for rapid, efficient adsorption of the phages and for maximal expression of target genes are given in Materials and Methods. An alternative solution to the problem of obtaining good adsorption might be to construct gene 1-containing phages that use receptors other than the lambda receptor, e.g. $\phi 80$.

Another potential problem in using these phages is that, under conditions of good adsorption, a sufficiently high multiplicity of infection completely inhibits all protein synthesis, as judged both by incorporation of [35 S]methionine and by accumulation of target gene product. This is illustrated by an experiment where cells containing plasmids with or without T7 promoters or target genes were pulse-labeled before infection or one hour after infection with an estimated 7, 14, 28 or 56 infective CE6 particles per cell. As shown in Figure 3, protein synthesis was completely abolished in five different cultures after infection by 56 phage particles per cell, and almost completely abolished in three of them after infection by 28 particles per cell. All of the cultures retained active protein synthesis at

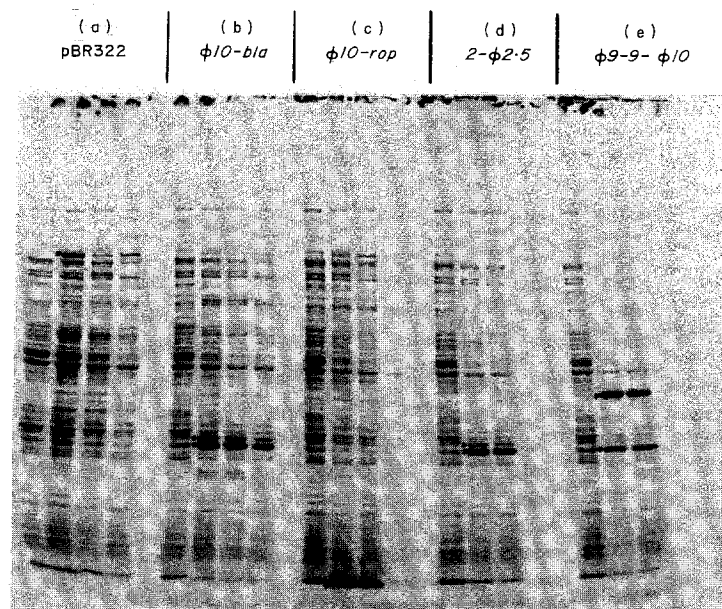


Figure 3. Effect of multiplicity of infection on protein synthesis. Cultures of HMS174 carrying different plasmids were grown in modified M9 medium containing 20 μ g ampicillin/ml and infected with CE6 as described in Materials and Methods, except that 10 mM-MgSO₄ was not added before infection. Immediately before or 1 h after infection, 50- μ l samples of culture were labeled for 5 min with 0.5 μ Ci [35 S]methionine. The cells were collected by centrifugation and the proteins subjected to electrophoresis in the presence of sodium dodecyl sulfate in a polyacrylamide gel containing a 10% to 20% (w/v) gradient of acrylamide and a 5% (w/v) stacking gel, followed by autoradiography, as described (Studier, 1973). From left to right in each set, lanes represent cells uninfected, or infected with approximately 7, 14, 28 or 56 infectious particles of CE6 per cell. The plasmids were: (a) pBR322, (b) pAR951, (c) pAR1494, (d) pAR219 and (e) pAR441. Where the plasmid contains any T7 promoters, T7 genes or $\phi 10$, the order of these is indicated above the lanes. Whenever T7 genes are present, transcription from the T7 promoter is directed counterclockwise, which also directs transcription of the *bla* gene of the plasmid (see Fig. 2). When no T7 gene is present, the plasmid mRNA that would be transcribed from $\phi 10$ is indicated, *bla* for counterclockwise transcription and *rop* for clockwise. The positions of individual proteins are marked by dots to the left of the lane representing cells infected at a multiplicity of 7. T7 RNA polymerase is made in all infections; its position is marked in set (a). The proteins made under direction of the T7 promoter in the plasmid are: (a) none; (b) β -lactamase; (c) *rop* protein; (d) β -lactamase and gene 2 protein (but gene 2 protein is not labeled because it has no methionine); (e) gene 9 protein and β -lactamase. The sizes of these proteins are given in Table 2.

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multiplicities of 7 and 14, and achieved high rates of synthesis of proteins whose genes are transcribed by T7 RNA polymerase. The apparent difference in the multiplicity needed to abolish protein synthesis in the different cultures may reflect uncertainties in the cell titers, which were estimated in this experiment by measuring the absorbance of the cultures. Other experiments (not shown) determined that the inhibition of protein synthesis at high multiplicities is complete within five minutes of adding the phage. Because of this effect, optimal expression of target genes is achieved at multiplicities of 5 to 10, high enough to infect almost every cell but low enough that protein synthesis is not inhibited.

(ii) Kinetics of target protein synthesis

Initially, we had considered that it would be necessary or desirable to eliminate competition from lambda or host mRNAs in order to direct the maximum resources of the cell to the production of the target protein. Transcription of lambda DNA can be repressed by using a host that is lysogenic for a phage of the same immunity, and competing transcription of both host and lambda DNA could be inhibited by producing T7 gene 2 protein or adding rifampicin, which inhibit *E. coli* but not T7 RNA polymerase (Hesselbach & Nakada, 1977; Chamberlin *et al.*, 1970). (Rifampicin would not be useful with HMS174, which has a resistant RNA polymerase.) However, it soon became apparent that T7 RNA polymerase is so active that strategies to prevent transcription of lambda or host DNA, or to prevent lysis by using the *Sam7* mutation, are unnecessary.

The time-course of protein synthesis upon CE6 infection of HMS174/pAR441 is shown in Figure 4. The rate of synthesis of gene 9 protein and β -lactamase begins to increase 10 to 15 minutes after infection, and by 15 to 20 minutes after infection these proteins are the most rapidly synthesized in the cell. Their rate of synthesis continues to increase until at least 30 minutes after infection and remains at a very high level for at least another 90 minutes. During this period, the rate of synthesis of host proteins gradually declines, and there is little evidence for synthesis of lambda proteins. Synthesis of T7 RNA polymerase can be detected but remains at a relatively low level; apparently, large amounts of T7 RNA polymerase are not needed in order to direct most of the protein synthetic capacity of the cell to the production of target proteins. The rate of synthesis of gene 9 protein is considerably higher than that of β -lactamase, even though there is every indication that the two mRNAs are produced in comparable amounts and are comparably stable (see below). We consider it likely that the difference in rate is due primarily to a difference in efficiency of translation.

Similar experiments (not shown) have examined stimulation of synthesis of gene 9 protein and β -lactamase after infection of HMS174/pAR441 by D69, DE1, DE2, DE3 and DE4. (See Fig. 1 for the

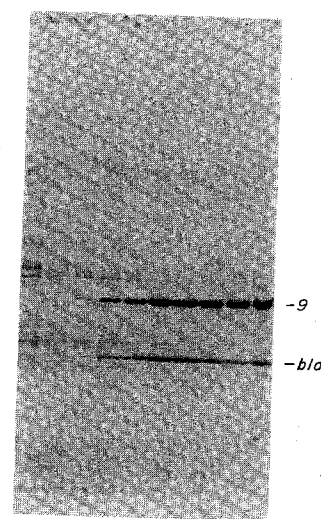


Figure 4. Time-course of protein synthesis after infection of HMS174/pAR441 by CE6. A growing culture of HMS174/pAR441 was infected with approximately 14 infectious particles of CE6/cell, samples were labeled for 5 min with [35 S]methionine, and the labeled proteins were analyzed, all as described in the legend to Fig. 3. More than 99% of the cells in the culture were infected within 6 min, as indicated by loss of ability to form colonies. From left to right in the Figure, lanes represent samples that were labeled beginning at infection, and 5, 10, 15, 20, 30, 45, 60, 90 and 120 min after infection. The positions of gene 9 and *bla* proteins are indicated.

location of gene 1 relative to *E. coli* promoters in these phages.) As expected, no stimulation of target protein synthesis is observed after infection by D69, which cannot supply any T7 RNA polymerase; nor is any stimulation observed after infection by DE1, a result indicating that little if any transcription of gene 1 occurs during infection by DE1. Stimulation by DE2, on the other hand, is comparable to that by CE6, as is stimulation by DE3 or DE4 in the presence of IPTG. In the absence of inducer, DE3 stimulates some target protein synthesis, but not as much as in the presence of inducer: apparently, the infecting copies of the *lacUV5* promoter are only partially repressed. Likewise, DE4 stimulates considerably less target protein synthesis in the absence of inducer than in its presence or than is stimulated by DE2 (which contains no *lacUV5* promoter): apparently, repression of the *lacUV5* promoter in DE4 blocks transcription initiated at p_L and/or p_i . Such blocking of transcription from an upstream promoter by repressed *lacUV5* promoter has been reported by Nakamura & Inouye (1982). Finally, when DE2 infects an immune host, target protein synthesis is delayed by a few minutes and may not reach quite as high a level as in a sensitive host, but very substantial production of target protein occurs none the less. In an immune host, p_L should be repressed: perhaps more DNAs enter the cell than can be repressed by the *cI* protein present at the time of infection, or perhaps transcription from the p_i promoter, which is known to function at

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These results show clearly that T7 RNA polymerase is capable of focusing the resources of the cell on the production of target proteins under control of a T7 promoter in a plasmid. The extent of the response depends on the amount of T7 RNA polymerase present, but relatively small amounts appear to generate a large response. The rate of synthesis of the target protein can be much higher than the rate of synthesis of any host protein, and such high rates can continue for three hours (Fig. 7) or longer (not shown).

(iii) Transcription from target promoters in plasmids

Transcription during infection by CE6 was analyzed by pulse-labeling with $^{32}\text{PO}_4$ at different times after infection, followed by electrophoresis on agarose gels (Fig. 5). In each case, the most prominent RNAs labeled immediately before infection were the 23 S and 16 S ribosomal RNAs and their immediate precursors. For size reference, mature 23 S rRNA is 2904 nucleotides long and mature 16 S rRNA is 1542 nucleotides long (Brosius *et al.*, 1981). When the host carried pBR322, labeling of ribosomal RNA continued for about 30 minutes, after which it decreased, presumably because of the phage infection (Fig. 5(a)). In contrast, when the host carried a plasmid having a

T7 promoter directed counterclockwise (Fig. 5(b)) or clockwise (Fig. 5(c)), labeling of ribosomal RNAs diminished markedly by 10 to 15 minutes after infection, and label began to be incorporated into RNAs having a broad distribution of sizes, including those considerably larger than ribosomal RNAs. Within the broad distribution of sizes are discrete bands of RNA. The apparent rate of synthesis of these RNAs continued to increase until at least 60 minutes after infection. That these large molecules are RNA has been confirmed by their sensitivity to RNase or alkali.

The distribution of RNAs produced when the plasmid carries a T7 promoter is similar to that produced by T7 RNA polymerase *in vitro*, where transcription counterclockwise can proceed around the plasmid several times with only weak termination at multiple sites, and where transcription clockwise can proceed around the plasmid several times, but with more efficient termination at two or three specific sites (McAllister *et al.*, 1981). The strongest clockwise termination site observed *in vitro* appears to be near nucleotide 3080 of pBR322 DNA, where a potential 11 base-pair stem-and-loop structure ends in seven T residues (Sutcliffe, 1979). Termination at the first, second or third encounter with this termination site in pAR1494 would produce discrete RNAs about 2750, 7200 and 11,700 nucleotides long. In the experiment of Figure 5(c), bands of about these sizes first

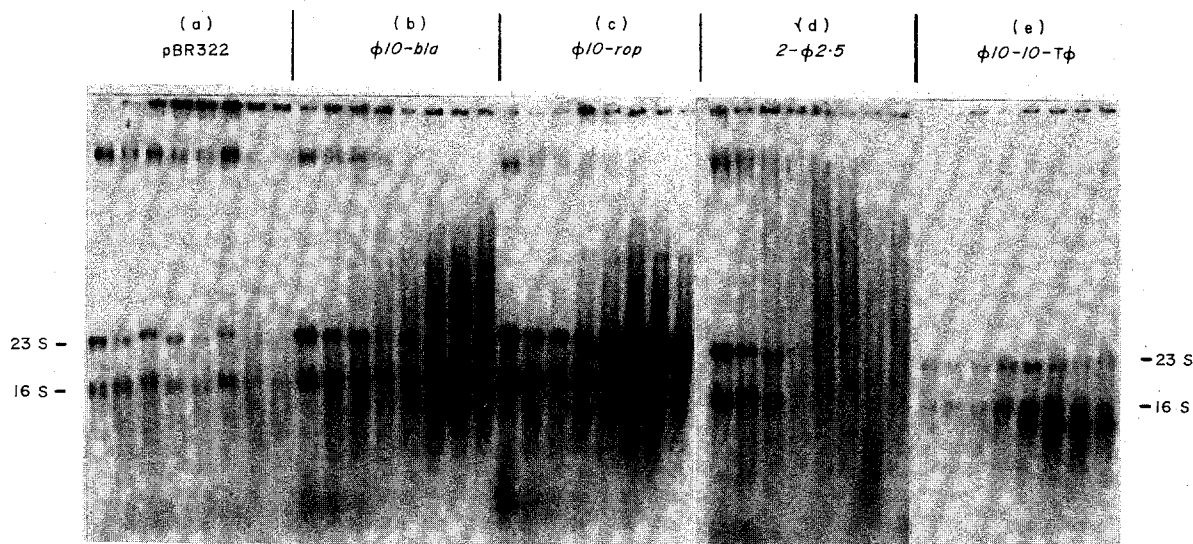


Figure 5. Time-course of RNA synthesis after infection of different plasmid-carrying cultures by CE6. Cultures of HMS174 carrying different plasmids were grown in modified B2 medium containing 20 μg ampicillin/ml and infected with approximately 7 infectious particles of CE6/cell, as described in Materials and Methods. Samples of culture were labeled for 5 min with $^{32}\text{PO}_4$ (25 $\mu\text{Ci}/\text{ml}$), and the cells were collected by centrifugation and suspended in an equal volume of 10 mM-sodium phosphate (pH 7.0), 2 mM- Na_3EDTA , 1% (w/v) sodium dodecyl sulfate, 1% (v/v) β -mercaptoethanol, 10% (v/v) glycerol, 0.01% (w/v) bromophenol blue. Each sample was mixed with 1/9 vol. 37% (v/v) formaldehyde, placed in a boiling waterbath for 2 min, and then an amount equivalent to 5 μl of culture was subjected to electrophoresis in a 1% (w/v) agarose gel containing 50 mM-sodium phosphate (pH 7.0), 2 mM- Na_3EDTA , 0.1% sodium dodecyl sulfate, followed by autoradiography. From left to right in each set, lanes represent samples labeled beginning 5 min before, and 0, 5, 10, 15, 30, 45 and 60 min after infection. The plasmids were: (a) pBR322, (b) pAR951, (c) pAR1494, (d) pAR219 and (e) pAR436. Genes under control of T7 promoters in the plasmid are indicated above each set, as described in the legend to Fig. 3. The positions of 23 S and 16 S ribosomal RNAs are indicated.

appeared about 10 to 15 minutes after infection, and by 60 minutes after infection the amount of label being incorporated into the 2750-nucleotide RNA was approaching the amount incorporated into ribosomal RNAs before infection.

Transcription in the counterclockwise direction appears to produce somewhat smaller amounts of a discrete RNA slightly larger than 16 S precursor RNA (Fig. 5(b)), suggesting that a weak termination or processing event may occur *in vivo*. A possible place for such termination or processing might be a cluster of three potential stem-and-loop structures that end with runs of T residues near nucleotides 3208, 3108 and 3048 of pBR322, which would produce RNAs about 1575, 1675 or 1735 nucleotides long.

In these experiments, no inhibitors of *E. coli* RNA polymerase were added or known to be produced, yet the rate of RNA synthesis by *E. coli* RNA polymerase decreased drastically as the rate of synthesis by T7 RNA polymerase increased. It seems likely that T7 RNA polymerase is so active that it simply outcompetes the host enzyme for precursors. In doing this, it reduces the synthesis of its own mRNA, a sort of feedback that presumably limits the amount of T7 RNA polymerase that can be accumulated.

RNA synthesis was also analyzed after infection of a plasmid carrying T7 gene 2 (Fig. 5(d)), which specifies an inhibitor of *E. coli* RNA polymerase (Hesselbach & Nakada, 1977). In this plasmid, the T7 promoter is behind the gene and transcription must proceed all the way around the plasmid in order to make gene 2 mRNA, but T7 RNA polymerase is known to do this (Studier & Rosenberg, 1981; McAllister *et al.*, 1981). Production of gene 2 protein should completely inhibit *E. coli* RNA polymerase, thereby shutting off synthesis of all host and lambda RNAs, including gene 1 mRNA. Indeed, incorporation into ribosomal RNAs had stopped by ten minutes after infection (except for a barely detectable amount that is presumably due to uninfected cells) (Fig. 5(d)). Again, a heterogeneous distribution of RNAs appeared, the expected products of T7 RNA polymerase. These continued to be made until at least 60 minutes after infection, although the rate did not continue to increase as it did when *E. coli* RNA polymerase was not inactivated (Fig. 5(b)). The rate may in fact have decreased after 30 minutes (although it seems likely that the 45-minute sample suffered some degradation during sample preparation). In any case, T7 RNA polymerase continues to be quite active for at least 20 minutes after *E. coli* RNA polymerase has been inactivated, and probably for much longer. It seems likely that T7 RNA polymerase is quite stable in the cell and does not have to be continually replenished to be effective.

Plasmid pAR436 contains a fragment of T7 DNA having the $\phi 10$ promoter, gene 10, and T ϕ , the natural terminator for T7 RNA polymerase, oriented so that transcription from $\phi 10$ is directed

counterclockwise. The transcript from $\phi 10$ to T ϕ is 1307 nucleotides, somewhat smaller than 16 S ribosomal RNA. Incorporation into this band began 10 to 15 minutes after infection and continued to increase until at least 60 minutes after infection, when incorporation into this band was at least as rapid as into ribosomal RNA before infection (Fig. 5(e)). Termination at T ϕ must be quite efficient: the band arising from readthrough of T ϕ and then termination after transcription once around the plasmid can be detected in the original film, but it is very weak relative to the band of 1307-nucleotide RNA produced by termination upon the first encounter with T ϕ .

In the patterns of Figure 5(e), synthesis of ribosomal RNAs did not decrease as rapidly or as drastically as that observed with the other $\phi 10$ -containing plasmids (Fig. 5(b) and (c)). It seems likely that termination at T ϕ reduces the competition with *E. coli* RNA polymerase sufficiently to allow significant ribosomal RNA synthesis to continue, an interpretation that would be consistent with the observations on RNA accumulation discussed below.

The RNAs produced from plasmids by T7 RNA polymerase accumulate to levels where they can be observed by ethidium bromide fluorescence in electrophoretic patterns of total RNA. The RNA patterns shown in Figure 6 were produced by induction (section (c), below), but similar results were obtained after CE6 infection (not shown). Where the plasmid insert contains only a T7 promoter (Fig. 6(b) and (c)), a considerable amount of RNA larger than ribosomal RNAs accumulates, whether the T7 promoter is directed counterclockwise or clockwise (compare Fig. 6(b) and (c) with (a)). Where the RNA contains a single efficient RNase-III cleavage site from T7 (Fig. 6(d)), the large, heterogeneous RNAs are cut at this site and accumulate as a discrete band of the length expected for transcription once around the entire plasmid. Within an hour after induction, the amount of RNA accumulated in this band approaches that in the ribosomal RNA bands. Where the plasmid DNA contains T ϕ (Fig. 6(e)), little RNA larger than ribosomal RNA accumulates, as expected if T7 RNA polymerase terminates transcription efficiently at T ϕ . A substantial amount of the RNA that terminates at T ϕ accumulates, but it migrates just ahead of 16 S ribosomal RNA and is partially obscured by it in Figure 6(e). Other experiments (not shown) have resolved a discrete $\phi 10$ -T ϕ RNA band more clearly. The parallel uninduced culture corresponding to each of the induced cultures represented in Figure 6 gave RNA patterns essentially identical with those in Figure 6(a), where the plasmid has no T7 promoter (not shown).

The turbidities of the induced and parallel uninduced cultures in the experiments shown in Figure 6 were measured as a function of time after induction (not shown). Where the plasmid carried no T7 promoter (Fig. 6(a)), or carried $\phi 10$ -T ϕ

-23 S
-16 S

Fig. 6. Cultures of uninduced and infected cultures were labeled in an equal volume, 1% (v/v) of vol. 37% (v/v) was subjected to 0.1% EDTA, 0.1% samples labeled 22, (b) pAR951, labeled above each

using our collection of cloned T7 genes. Examples of pulse-labeled proteins detected by gel electrophoresis and autoradiography are given in Figures 3, 4 and 7. Proteins have been observed by enzymic activity and as stained bands in gel electrophoretic patterns of total cell proteins similar to those given in Figure 8 (where T7 RNA polymerase was supplied by induction of chromosomal gene 1). In almost all cases, the known or expected T7 protein was observed, but the rate of synthesis of the protein and the level to which it accumulated varied over a wide range. In general, the relative rate of synthesis and the level of accumulation of individual proteins parallels the relative rate of synthesis observed during T7 infection (Dunn & Studier, 1983). Taking into account the results of the previous section, it seems likely that the T7 expression system produces saturating amounts of mRNA, and that the relative rates of synthesis reflect mainly the relative efficiencies of translation of the different mRNAs.

Consider first the protein patterns of Figure 3. When the host cell contains pBR322, which has no T7 promoter, the new bands that appear after infection must be proteins specified by the infecting phage (Fig. 3(a)). Among the strongest bands appearing is that representing T7 RNA polymerase, identified by its relative mobility. When the plasmid contains a T7 promoter, labeling of proteins specified by the phage (including T7 RNA polymerase) is much diminished, consistent with the decrease in RNA synthesis by *E. coli* RNA polymerase and translational competition from the flood of transcripts from the plasmid, as noted in the previous section. When the T7 promoter is directed counterclockwise (Fig. 3(b)), β -lactamase is synthesized at a high rate after infection and appears as a triple band, representing the unprocessed precursor and two conformers of the processed protein (Koshland *et al.*, 1982). When the T7 promoter is directed clockwise (Fig. 3(c)), a rapidly migrating band which probably represents the *rop* protein of pBR322 (Cesareni *et al.*, 1982; Som & Tomizawa, 1983), is heavily labeled, and labeling of β -lactamase is diminished. Apparently, active transcription in the reverse direction interferes with synthesis and/or translation of the *bla* mRNA, as reported for other genes (Coleman *et al.*, 1984). The target genes we have inserted into pBR322 have usually been oriented so that they are expressed in the counterclockwise direction.

When the plasmid contains gene 2, β -lactamase is labeled intensely one hour after infection (Fig. 3(d)), even though newly made gene 2 protein would have shut off all transcription by *E. coli* RNA polymerase around ten minutes after infection (Fig. 5(d)). Consistent with the transcription pattern (Fig. 5(d)), it appears that the T7 RNA polymerase and/or stable gene 1 mRNA that is accumulated in the period before *E. coli* RNA polymerase is inhibited is sufficient to direct active gene expression from the plasmid for some time. Gene 2 protein is not labeled in these patterns

because it contains no methionine (Dunn & Studier, 1983), but it accumulates to levels where it can be identified as a band in electrophoresis patterns of total cell proteins stained with Coomassie brilliant blue (not shown). When the plasmid contains gene 9 (Fig. 3(e)), both gene 9 protein and β -lactamase are made.

Additional examples of target protein synthesis after CE6 infection are given in Figure 7. For each of these experiments, fewer than 4% of the cells remained uninfected six minutes after CE6 was added to the growing cultures, so the background of label incorporated into uninfected cells was minimal. The proteins were pulse-labeled with [35 S]methionine immediately before, or at one, two or three hours after infection. As expected, β -lactamase was produced in all of these infections, the least in Figure 7(e), where the presence of T ϕ in the plasmid reduces transcription of the *bla* gene. The T7 proteins identified in these patterns are: gene 4-7 protein (unknown function) and gene 5 protein (DNA polymerase) in Figure 7(a); gene 6 protein (exonuclease) and gene 6-5 protein (unknown function) in Figure 7(b); gene 8 protein (head-tail junction) and a fragment of gene 9 protein (head assembly) in Figure 7(c); gene 9 protein in Figure 7(d); and gene 10A and gene 10B proteins (head proteins) in Figure 7(e). The gene 10B protein is produced by frameshifting during translation of the gene 10A protein (Dunn & Studier, 1983).

In all of these infections, the target proteins were produced at substantial rates and synthesis of host proteins was greatly reduced. This shift in gene expression reflects the shift in mRNA population in favor of transcripts produced by T7 RNA polymerase, and the relatively high translational efficiency of the T7 mRNAs. The high rates of synthesis of target proteins continued for at least three hours, except for the gene 6 and gene 6-5 proteins, where the rate decreased significantly in the second and third hours. Perhaps this decrease was caused by the action of gene 6 protein, which is an exonuclease, or gene 6-5 protein, whose function is unknown.

By three hours after infection with CE6, all of the T7 proteins identified in Figure 7 accumulate to a level where they can be identified in gel electrophoresis patterns of total cell proteins stained with Coomassie brilliant blue (not shown, but see Fig. 8). Clearly, T7 RNA polymerase is capable of directing the accumulation of substantial amounts of a variety of target proteins in a relatively short time.

In some cases, where no *E. coli* protein obscures it, a small amount of target protein can be seen to be labeled in uninfected cells in the complete absence of T7 RNA polymerase. Such labeling is apparent for the gene 9 protein in Figures 3(e) and 7(d), and for the gene 5, 8 and perhaps gene 10 proteins in Figure 7. In some of these cases, weak *E. coli* promoters have been identified ahead of the gene in the cloned fragment, and these weak promoters almost certainly contribute to expression

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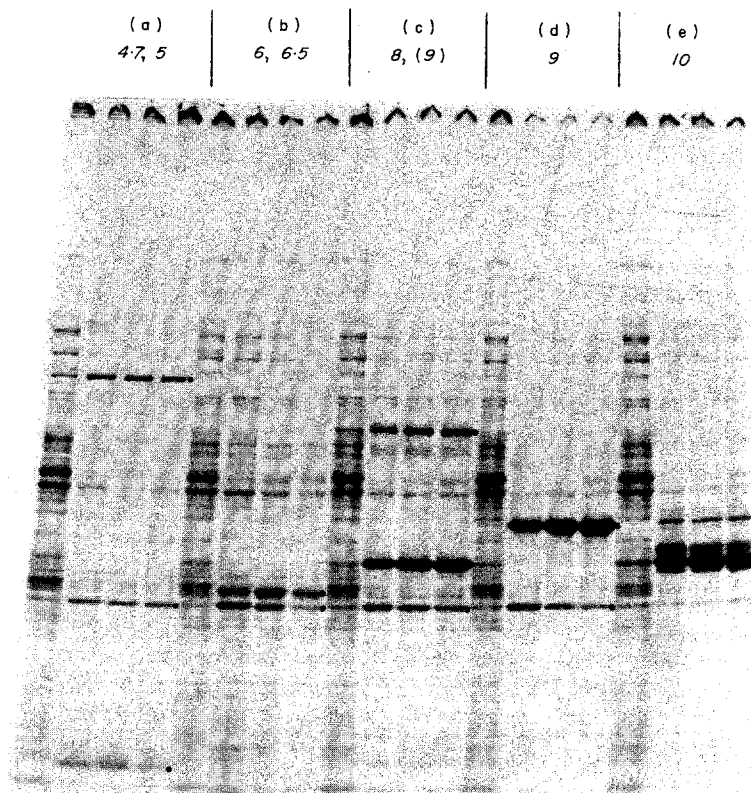


Figure 7. Time-course of protein synthesis after infection of different plasmid-carrying cultures by CE6. Cultures of HMS174 carrying different plasmids were grown, infected with approximately 8 to 12 infectious particles of CE6/cell (except 25/cell for pAR1012), labeled for 5 min with [35 S]methionine, and the labeled proteins analyzed, all as described in the legend to Fig. 3. In each culture, more than 96% of the cells were infected within 6 min, as indicated by loss of ability to form colonies. From left to right in each set, lanes represent samples labeled immediately before, and 1, 2 and 3 h after infection. The plasmids were: (a) pAR1012, (b) pAR525, (c) pAR213, (d) pAR441 and (e) pAR436. The T7 proteins expressed under direction of T7 promoters in the plasmid are indicated above each set of lanes and marked by dots in the Figure; both the gene 10A and gene 10B proteins are marked. β -Lactamase is also expressed from T7 promoters in all of these plasmids, and its position is also marked in each set. The sizes of these proteins are given in Table 2, except for the fragment of gene 9 protein in set (c), which is predicted to contain 243 amino acid residues.

in the absence of T7 RNA polymerase. However, it is clear that genes can be expressed at levels sufficient to complement T7 mutants even when no *E. coli* promoter lies ahead of the gene in the cloned fragment (Davanloo *et al.*, 1984; and unpublished results).

(c) *Selective expression of target genes when T7 RNA polymerase is provided by induction of a chromosomal copy of the gene*

Infection by CE6 or DE2 will provide enough T7 RNA polymerase to transcribe target genes in a plasmid at very high rates, and even genes that are very toxic to *E. coli* can be expressed in this configuration. However, induction of a copy of gene 1 that is already in the cell can be more convenient than infection to provide T7 RNA polymerase. The main problem with induction is that the basal level of T7 RNA polymerase present in uninduced cells must be sufficiently low that target plasmids can be maintained in the same cell.

The more toxic the target gene, the lower the basal level of T7 RNA polymerase required. On the other hand, the cell must be capable of inducing sufficient T7 RNA polymerase to transcribe the target plasmid at high level.

We have used lysogens of DE1, DE2, DE3 and DE4 to explore both how well gene 1 can be shut off when carried in the chromosome and how well it can provide T7 RNA polymerase for expressing target genes from plasmids in the same cell. Lysogens of both HMS174 and BL21 were made but we have used mainly the latter.

(i) *Using a gene 1 deletion mutant to test for active T7 RNA polymerase*

A sensitive test for the presence of active T7 RNA polymerase in a cell is the ability of a culture to support plaque formation by a deletion mutant of T7 that completely lacks gene 1. For this purpose, we have used deletion 4107, described in Materials and Methods. The cloned gene 1-containing fragment lies entirely within the

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These are clearly that whether in or under co lower basal single-copy pAR1219 c

segment deleted, so the frequency of a phage picking up an active gene *I* by recombination with the chromosomal copy is extremely low and has not been detected ($<10^{-9}$).

Plaque formation by 4107 is absolutely dependent on active T7 RNA polymerase supplied by the host: when 4107 is plated on a lawn of BL21 or BL21(DE9), no plaques develop even after a long incubation. BL21(DE2), on the other hand, provides levels of T7 RNA polymerase appropriate for normal growth, and 4107 gives normal plaques and makes lysates of normal titer when grown on BL21(DE2). Expression of gene *I* in BL21(DE2) is due to transcription from the p_i promoter (see Fig. 1), which is known to remain active in the prophage (Shimada & Campbell, 1974).

On the other hand, BL21(DE1), which has gene *I* in the opposite orientation from BL21(DE2), plates 4107 very poorly: the plaques take a long time to develop, are very small, and appear with only 0.1 to 0.5 times the normal efficiency. By these indications, lysogens of DE1 have the least T7 RNA polymerase activity of any of the gene *I*-containing strains tested. Apparently, integration of DE1 into the chromosome did not place gene *I* under control of an active *E. coli* promoter, although a very small amount of mRNA is being produced.

DE3 has gene *I* in the same orientation as DE1 but, in addition, has the *lacI-lacUV5* fragment ahead of gene *I*. A lawn of BL21(DE3) plates 4107 somewhat better than does a lawn of BL21(DE1), but the plaques are still small, take considerably longer than normal to develop, and form with perhaps half the normal efficiency. The presence of the uninduced *lacUV5* promoter seems to have increased transcription of gene *I* somewhat, but the level of T7 RNA polymerase remains very low, much lower than is observed for a multicopy plasmid (Davanloo *et al.*, 1984). However, when BL21(DE3) is induced by adding IPTG to the plate T7 RNA polymerase is produced in quantities sufficient to enable 4107 to make normal plaques.

DE4 has gene *I* in the same orientation as DE2, i.e. expressed from the p_i promoter, but has the *lacI-lacUV5* fragment between p_i and gene *I*. In contrast to BL21(DE2), which plates 4107 normally, uninduced BL21(DE4) plates 4107 almost as poorly as BL21(DE3). The presence of the repressed *lacUV5* promoter seems to interfere with transcription of gene *I* from the P_i promoter in the prophage, perhaps by blocking the transit of transcribing *E. coli* RNA polymerases. This same effect was observed during infection by DE4 (see section (b)(ii), above). However, when BL21(DE4) is induced by adding IPTG to the plate, the 4107 plaques are normal.

These tests of the ability to plate 4107 show clearly that the same gene *I*-containing fragment, whether in the absence of known *E. coli* promoters or under control of the *lacUV5* promoter, generates lower basal levels of T7 RNA polymerase in the single-copy lysogen than in the multicopy plasmids pAR1219 or pAR1151. The induced levels of T7

RNA polymerase are also very different: upon induction by IPTG, cultures of BL21/pAR1219 accumulate perhaps 10% to 20% or more of the total protein of the cells as T7 RNA polymerase, whereas BL21(DE3) or BL21(DE4) produce an amount barely detectable in gel patterns of total protein, estimated to be perhaps 2% to 5% of the amount accumulated in BL21/pAR1219 (not shown). The ratio of the induced level of T7 RNA polymerase in the lysogens relative to the plasmid-containing strain is approximately the same as the ratio of copies of gene *I* in the two types of cell, and it seems likely that this ratio holds for the basal level in the uninduced state as well. Although induction of gene *I* in the lysogens produces much lower levels of T7 RNA polymerase than does induction of the multicopy plasmid, these amounts are sufficient for normal growth of T7 and, as shown in the next section, for active expression of target genes from plasmids. Of course, addition of IPTG has no effect on T7 RNA polymerase levels in BL21(DE1) or BL21(DE2), where gene *I* is not under control of the *lacUV5* promoter.

The ability of 4107 to form plaques is a useful and convenient test for the presence of active T7 RNA polymerase in cells. We presume that this test detects very low levels of T7 RNA polymerase, but we have not tried to determine the minimum concentrations of T7 RNA polymerase that can be detected.

(ii) Expression of target genes in BL21(DE3)

The BL21(DE3) lysogen provides an inducible source of T7 RNA polymerase having a basal level sufficiently low that most T7 genes can be maintained under control of a T7 promoter in a multicopy plasmid in this strain. However, as discussed below, some plasmids are relatively unstable in BL21(DE3) and others cannot be established at all, even though they can be established in BL21 itself.

Where plasmids can be maintained in BL21(DE3), substantial amounts of target RNAs and proteins can be produced after induction of gene *I* by IPTG. Figure 6 shows examples of RNA accumulation after induction (discussed in section (b)(iii), above); Figure 8 shows examples of protein accumulation. When BL21(DE3) carries pBR322, which has no T7 promoter, addition of IPTG has little effect on protein synthesis, except to stimulate some accumulation of both β -galactosidase and T7 RNA polymerase (Fig. 8(a)). When the plasmid contains a T7 promoter directed toward *bla*, both unprocessed and processed β -lactamase rapidly accumulate to high levels (Fig. 8(b)). When the plasmid carries T7 genes 2.5, 9 or 10 as well (Fig. 8(c), (d) and (e)), the T7 protein accumulates to very high levels, and can approach 50% or more of the total cell protein within three hours. Not much β -lactamase accumulates in these cells, probably because the *bla* mRNA does not compete well for translation against the T7 mRNAs and, in the case of gene 10 (Fig. 8(e)), because the presence

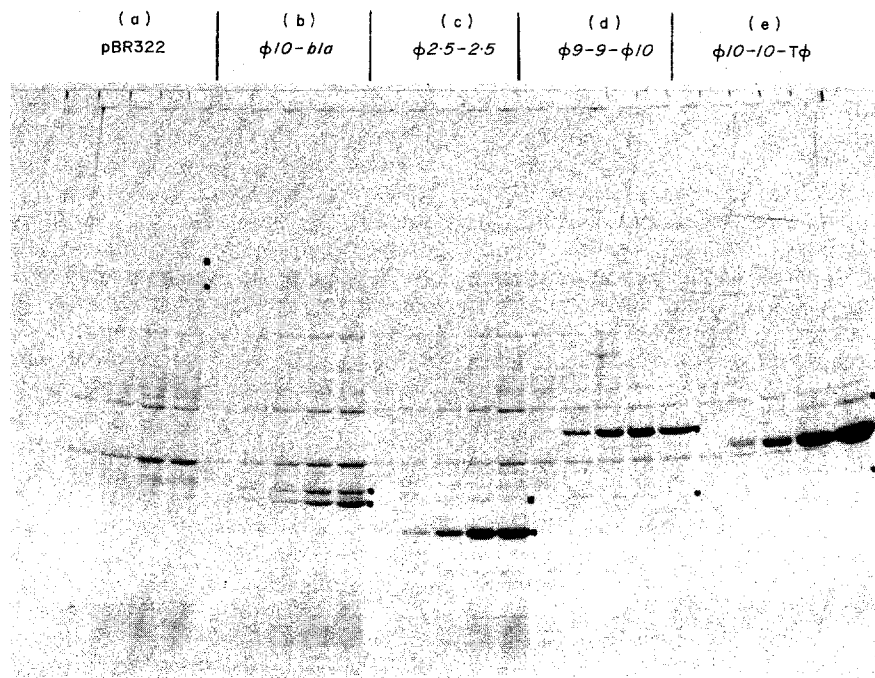


Figure 8. Accumulation of protein directed by T7 RNA polymerase. Cultures of BL21(DE3) carrying the indicated plasmids were grown in ZY medium containing 200 μ g ampicillin/ml, and synthesis of T7 RNA polymerase was induced by adding 0.4 mM-IPTG. The titer of samples of culture removed before IPTG was added showed that almost all of the cells had plasmid in each culture except that containing pAR511 (set (c)), where only about 87% of the cells had plasmid. Cells were collected by centrifugation, an amount equivalent to 10 μ l of culture was subjected to electrophoresis, as described in the legend to Fig. 3, and the proteins were visualized by staining with Coomassie brilliant blue. From left to right in each set, lanes represent samples collected immediately before, and 0.5, 1, 2 and 3 h after IPTG was added to the culture. The plasmids were: (a) pBR322, (b) pAR951, (c) pAR511, (d) pAR441 and (e) pAR436. Genes under control of T7 promoters in the plasmid are indicated above each set, as described in the legend to Fig. 3. The positions of β -galactosidase (higher) and T7 RNA polymerase (lower), whose synthesis is induced by IPTG, are marked by dots in set (a). The positions of the proteins expressed from the plasmid under control of T7 promoters are marked in the other sets, including β -lactamase in sets (b) to (e) and both the gene 10A and 10B proteins in set (e).

of T ϕ prevents much *bla* mRNA from being made. As when T7 RNA polymerase is introduced by CE6 infection, inducing synthesis of T7 RNA polymerase in BL21(DE3) can direct most of the resources of the cell to the production of a single target protein encoded in the plasmid.

At least some target protein would be expected to be present in the uninduced cells, due to the basal level of T7 RNA polymerase. Indeed, small amounts can be detected in the original of Figure 8, but the level is quite low relative to that of other proteins in the cell, or relative to the amount accumulated after induction.

(iii) Stability of target plasmids in BL21(DE3)

T7 RNA polymerase by itself is not toxic to *E. coli* cells: BL21(DE3) and BL21(DE4) grow as well in the continuous presence of IPTG as in its absence, and BL21/pAR1219, which accumulates a steady-state level of T7 RNA polymerase that is perhaps 20% of the cell protein, also continues to grow indefinitely in the presence of IPTG, although more slowly than in its absence. However, if the cell contains a plasmid with a T7 promoter, induction

of T7 RNA polymerase will usually kill the cell. The ability of a cell to tolerate both the gene for T7 RNA polymerase and T7 promoters depends on both the level of active T7 RNA polymerase in the cell and the effect on the cell of transcription from the T7 promoter.

The basal level of T7 RNA polymerase in uninduced BL21(DE3) is sufficiently low that derivatives of pBR322 having only a T7 promoter in the *Bam*HI site are stable, even when grown for many generations in the absence of ampicillin. Plasmids containing innocuous target genes under control of a T7 promoter are also stable. However, as the toxicity of the target gene increases, plasmids become more difficult to maintain; and if the target gene is sufficiently toxic, the plasmid cannot be established in BL21(DE3). In cases of intermediate toxicity, the plasmid can be established and maintained in the presence of ampicillin, but cells that have lost the plasmid will overgrow the culture more or less rapidly in the absence of selective pressure. To obtain maximum accumulation of target proteins, the great majority of cells in the culture must be competent to express the target

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gene at the time inducer is added. Unless one is careful, this will not always be the case.

Part of the problem in maintaining these unstable plasmids is that they produce and secrete so much β -lactamase that, at sufficiently high cell densities, all of the ampicillin in the medium will be destroyed. After this point is reached, cells without plasmid will be able to grow. Even when the growth medium contains 200 μ g ampicillin/ml, all of the ampicillin can be destroyed before the cells reach a concentration of a few times 10^7 /ml, as indicated by the observation that more than 99% of the viable cells had lost a relatively toxic plasmid by the time the culture has reached 5×10^7 cells/ml. The difference in growth rate between BL21(DE3) cells that do or do not contain this plasmid is large; the culture grows very slowly until all the ampicillin is destroyed, whereupon cells without plasmid rapidly overgrow the culture. For less toxic plasmids, the difference in growth rate is not so pronounced, and more than 95% of the cells will retain plasmid all of the way to saturation.

A related problem is that saturated cultures of cells containing pBR322 or its derivatives can accumulate sufficient β -lactamase that, even after dilutions of 100 to 200-fold or greater, into fresh medium containing 100 μ g ampicillin/ml, all of the ampicillin will be destroyed and cells without plasmid will be able to grow. This is not a problem when the plasmid is stable, but when the plasmid is unstable, cultures thought to contain plasmid simply because they were grown in medium that contained ampicillin can be almost completely free of plasmid.

Some target plasmids have a different kind of instability in BL21(DE3): they are stable in the logarithmic phase of growth, but when the culture reaches saturation, cells that contain the plasmid begin to die. T7 gene 3, which specifies an endonuclease, is one target gene that causes this behavior. Since all of the ampicillin will be destroyed by the time the culture reaches saturation, cells that lack plasmid can grow to replace the plasmid-containing cells that die. Incubation for several hours after saturation is reached can be sufficient to generate a culture in which most of the viable cells lack plasmid. Unless subcultures are inoculated at very high dilution, enough β -lactamase will be present to destroy the ampicillin in the fresh medium, and most of the cells that grow in the new culture will lack plasmid.

Because of these potential problems in maintaining target plasmids in BL21(DE3), we routinely sample cultures immediately before induction, to determine the fraction of cells that retain an inducible target plasmid, as described in Materials and Methods. BL21(DE3) carrying a plasmid with an active T7 promoter will typically be unable to make colonies in the presence of IPTG, but two exceptions to this plating behavior have been encountered. When a T7 promoter directs transcription clockwise from the *Bam*HI site of pBR322, opposite to the direction we have used for

most target genes, almost all cells give rise to a colony in the presence of IPTG. However, the cells that grow have lost the plasmid, and colonies do not appear if the plates contain ampicillin as well as IPTG. Apparently, clockwise transcription T7 RNA polymerase causes rather efficient segregation of cells that have lost plasmid, perhaps by interfering with replication and/or partition functions (Itoh & Tomizawa, 1980; Cesareni *et al.*, 1982; Som & Tomizawa, 1983). The second exception is when T7 gene 3.5 is under control of a T7 promoter in the plasmid. T7 lysozyme, the product of gene 3.5, inhibits transcription by T7 RNA polymerase (Moffatt & Studier, unpublished results), and this allows the induced cell to grow. Other exceptions may be encountered, but in cases where it is applicable, inability to form colonies in the presence of IPTG remains a simple and useful test for the presence of a functional target plasmid in BL21(DE3).

Where plasmids contain highly toxic genes, mutants that retain the plasmid but have lost the ability to induce the target gene can arise during growth of the uninduced culture. Such mutants can be recognized because they make colonies on plates containing both ampicillin and IPTG. However, in making this test, the culture must be diluted sufficiently so that the large amounts of β -lactamase that can be produced upon induction (e.g. see Figs 3(b) and 8(b)) do not destroy all of the ampicillin in the plate and allow cells without plasmid to grow. For typical target plasmids, this means that fewer than about 2×10^4 cells should be put on a plate and, for pAR951, which produces very high levels of β -lactamase, fewer than 2×10^3 cells per plate.

The problems of plasmid instability can be minimized by appropriate precautions, such as being sure that most cells in the culture frozen for storage do in fact contain inducible target plasmid, and that new cultures are grown either from sufficiently high dilution or from a colony that had been grown on a plate containing ampicillin. However, if a target gene is too toxic to be maintained in BL21(DE3), it can still be turned on by delivering gene 1 to the cell by infection.

4. Discussion

T7 RNA polymerase from the cloned gene is very efficient and selective in transcribing DNA in the *E. coli* cell. Only small amounts of T7 RNA polymerase are needed to generate very large amounts of RNA from DNA that is linked to a T7 promoter in derivatives of pBR322, a multicopy plasmid. This high rate of transcription by T7 RNA polymerase seems to interfere with transcription by *E. coli* RNA polymerase, thereby reducing expression of genes under the control of *E. coli* promoters, whether in chromosomal or in lambda DNA. Since there is little competition for template between the two polymerases, the interference seems likely to be due to competition for RNA

precursors. In favorable circumstances, essentially all of the resources of the cell can become concentrated on the production of an individual protein, which can accumulate rapidly to reach 50% or more of the total cell protein.

T7 RNA polymerase seems to transcribe DNA in the cell in much the same way as the purified enzyme transcribes purified DNA. When the plasmid contains the natural terminator for T7 RNA polymerase from T7 DNA (T ϕ), most of the transcription appears to stop at this site, although a small fraction of the polymerases read through and terminate after having transcribed completely around the plasmid. In the absence of T ϕ , most of the label is found in a broad distribution of sizes between about 1000 and 7000 base-pairs, but some of the molecules appear to reach 12,000 base-pairs or even larger, corresponding to transcription three or more times around the plasmid DNA without terminating. When the plasmid contains a unique RNase-III cleavage site from T7, RNA of length corresponding to transcription once around the plasmid can accumulate to about the same level as that of ribosomal RNA.

The distribution of sizes of the RNAs produced from plasmids that lack T ϕ or an RNase-III cleavage site seems somewhat smaller *in vivo* (Figs 5 and 6) than *in vitro* (McAllister *et al.*, 1981). Whether this is due to differences in labeling and analysis of the RNAs, degradation of the RNA *in vivo*, or something else has not been determined. However, the simple observation that these RNAs accumulate to levels apparent in total cellular RNA suggests that they are relatively stable *in vivo*. As discussed in Results, some of this apparent stability may simply be because the RNAs are so long that it takes a long time for exonucleases to digest them, and termination at T ϕ or cleavage at an RNase-III cleavage site that leaves a stem-and-loop structure at the 3' end of the RNA might further increase stability. It is also conceivable that T7 RNA polymerase may be able to produce mRNA so rapidly that the capacity of the cell to degrade it becomes overloaded.

The large amounts of mRNA generated by T7 RNA polymerase seem likely to be enough to saturate the protein-synthesizing apparatus of *E. coli*. If so, the rate of protein synthesis achieved with individual mRNAs should be a measure of the absolute translational efficiency of that mRNA. (Of course, other factors can limit the accumulation of a protein, such as instability of the protein in *E. coli* or an activity that would interfere with protein synthesis.) The rate of synthesis and extent of accumulation of individual T7 proteins vary over a wide range, and the relative rate of synthesis in the expression system correlates well with relative rate of synthesis during T7 infection. Translational efficiency is probably one of the most important determinants of how much protein can be produced, and the T7 expression system should be useful in testing what features of an mRNA determine its efficiency of translation. In most of the plasmid

configurations we use, T7 RNA polymerase produces mRNA for both β -lactamase and the protein of interest, so production of β -lactamase provides an internal control for assessing relative translational efficiencies.

T7 RNA polymerase seems to be quite efficient at transcribing almost any DNA linked to a T7 promoter, and high-efficiency termination signals seem to be rare. Purified T7 RNA polymerase, besides being very active on T7 DNA itself, has made complete transcripts of every DNA we know to have been tested so far, including molecules as large as 10,000 base-pairs from a variety of prokaryotes and eukaryotes (J. J. Dunn, personal communication). Our results indicate that the same is likely to be true *in vivo*. Thus, T7 RNA polymerase inside cells should be able to direct the production of large amounts of mRNAs, rRNAs, tRNAs and other RNAs from a variety of sources, either the natural sequence or its complement. Although all of the examples we have given here are for RNAs and proteins naturally adapted for synthesis in *E. coli*, we and others (unpublished results) have used this system to express genes from a variety of sources, including eukaryotes, and the expected RNA and/or protein has been produced in every case. The ability to produce high levels of complementary RNA may be useful in selective suppression of gene expression (Simons & Kleckner, 1983; Coleman *et al.*, 1984; Izant & Weintraub, 1985).

Once T7 RNA polymerase is present in the cell, T7 gene 2 protein or rifampicin can be used to inactivate *E. coli* RNA polymerase, so that all transcription in the cell will be due to T7 RNA polymerase. Initially, we had thought this might be useful for obtaining high-level expression, but target mRNAs are produced so actively by T7 RNA polymerase that we found little if any enhancement in the production of target proteins when rifampicin was added an hour after induction of gene 1. However, rifampicin does prevent continued growth of cells that have lost plasmid, thereby reducing the relative amounts of other cell proteins accumulated during long incubations. Addition of rifampicin can be convenient or useful in reducing the background in pulse-labeling experiments to identify RNAs or proteins whose synthesis is directed by T7 RNA polymerase (although this has not been necessary in most of the cases we have analyzed).

The T7 expression system is ideal for maintaining and expressing genes whose products are toxic to *E. coli*. T7 RNA polymerase and *E. coli* RNA polymerase recognize completely different promoter sequences (Dunn & Studier, 1983; Hawley & McClure, 1983), so it should be possible to place a gene under control of a T7 promoter without enhancing its expression by *E. coli* RNA polymerase. Since the toxic gene never has to be transcribed by *E. coli* RNA polymerase, sites for it could be found or prepared where transcription by *E. coli* RNA polymerase is as low as it is possible to

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Rather toxic genes can be tolerated in the *Bam*HI site of pBR322, but some transcription across this site does occur. Cloned gene *I* provides a very sensitive probe to look for, or to construct, specific cloning sites in *E. coli* where transcription by *E. coli* RNA polymerase is at a very low level. Its mRNA appears to be relatively stable and efficiently translated, and simple tests can detect vanishingly small amounts of T7 RNA polymerase activity. A sensitive test we have used is the ability of a lawn of cells to permit plaque formation by a T7 gene *I* deletion mutant. A potentially more sensitive test is the ability to tolerate a multicopy plasmid containing a toxic gene under control of a T7 promoter: the more T7 RNA polymerase in the cell, the more difficult it is to establish or maintain such plasmids; and the more toxic the gene in the plasmid, the more sensitive will be the test. Potentially, the most sensitive test of all for lack of expression would be whether a site could tolerate a copy of gene *I* under control of a T7 promoter: any expression would lead to an autocatalytic increase in the amount of T7 RNA polymerase that would almost certainly be lethal to the cell (Davanloo *et al.*, 1984). Using the first two tests, we have found that transcription from left to right across the *Bam*HI site of the lambda cloning vector D69 is at very low levels both during infection and in the prophage, and that placing a repressed *lacUV5* promoter ahead of the gene in this site increases its transcription. Much less expression is observed in this site in the single-copy prophage than in the *Bam*HI site of the multicopy plasmid pBR322.

When trying to accumulate large amounts of target protein, it is often more convenient to generate T7 RNA polymerase by induction rather than by infection. However, induction cannot be used if the target gene is so toxic that the basal level of T7 RNA polymerase in the uninduced cell is too high to allow the plasmid to be established. The lower the basal level of T7 RNA polymerase, the higher the toxicity of target gene that can be tolerated in the plasmid. When gene *I* is present as a single copy in the chromosome under control of the *lacUV5* promoter in the BL21(DE3) lysogen, basal levels are sufficiently low that most but not all T7 genes can be tolerated as target genes. Some plasmids can be established but are unstable in this host, and special care may be needed to ensure that most cells in the culture contain a functional target plasmid at the time of induction.

Several strategies could be considered for reducing the basal level of T7 RNA polymerase even lower than that in BL21(DE3). One possibility would be to search for inducible *E. coli* promoters that have basal levels of expression lower than the *lacUV5* promoter. Since high levels of T7 RNA polymerase are not needed to produce very high levels of target protein, the inducible promoter may not need to be particularly strong. Another

possibility would be to place a transcription terminator or attenuator between the inducible promoter and gene *I*, an approach used by Tabor & Richardson (1985). Other strategies might include reducing the activity of the T7 RNA polymerase present in the uninduced cell, by the use of a specific inhibitor such as T7 lysozyme (Moffat & Studier, unpublished results), or by blocking access to the T7 promoter by placing repressor binding sites sufficiently close to it.

In the configurations we have used, gene *I* is induced or delivered to the cell in the presence of the target gene. Since high-level transcription by T7 RNA polymerase inhibits transcription by *E. coli* RNA polymerase, and gene *I* is under control of an *E. coli* promoter, only limited amounts of T7 RNA polymerase can be accumulated. A different configuration, where T7 RNA polymerase is first accumulated to the desired level in the cell and the target gene is then delivered by phage infection, would avoid this limitation and allow the effects of different levels of T7 RNA polymerase to be tested. The rate of transcription of the target gene could, presumably, be made very high, and background transcription of host or phage genes could be eliminated completely by inactivation of *E. coli* RNA polymerase and/or ultraviolet light irradiation of the host before infection. This procedure might be useful for producing a large burst of expression from a toxic gene before the toxic effects are fully manifest, and might have advantages for screening cloned DNA fragments for coding sequences able to be translated in *E. coli*, perhaps particularly those that produce unstable proteins or are translated inefficiently.

It seems quite possible that T7 RNA polymerase (and/or similar RNA polymerases from other T7-like phages) will be able to function in cell types other than *E. coli*, and could therefore provide the basis for selective, high-level transcription in a variety of cell types. T7 promoter sequences are sufficiently large that they are unlikely to be found in the typical cell genome, so transcription should remain selective. Introduction of target DNA sequences under control of a T7 promoter, together with a source of active T7 RNA polymerase, might well be able to generate large amounts of target RNA and protein. Different cell types will probably present different barriers to be overcome, but it seems reasonable to expect that expression systems based on T7 RNA polymerase could be worked out for bacteria closely related to *E. coli*, and for Gram-positive bacteria, yeast, higher eukaryotes, and in fact for any cells where suitable viruses, plasmids or other delivery systems are available or could be developed.

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